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(54) Title: PRETREATMENT WITH GROWTH FACTORS TO PROTECT AGAINST CNS DAMAGE

(57) Abstract

Methods for protecting neural tissue from the effects of aging, trauma, toxic insult, neurological diseases or disorders comprise administering growth factors to the neural tissue of a mammal at a time prior to the onset of trauma or the manifestations of neurological disease or aging. The growth factors induce multipotent neural stem cells and neural stem cell progeny to proliferate and generate new neural cells which provide supportive and protective roles against the effects of aging, trauma, toxic insult, neurological diseases or disorders.

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PRETREATMENT WITH GROWTH FACTORS TO PROTECT AGAINST CNS DAMAGE

Field of the Invention:

This invention relates to methods for protecting neural cells against injury and death, as a result of trauma, neurodegenerative disease processes, toxic insult, malfunction, or aging by providing factors to the central nervous system of a subject.

Background of the Invention:

Neurodegenerative diseases such as Parkinson's disease, Alzheimer's disease, Huntington's disease, multiple and amyotrophic lateral sclerosis, stroke, schizophrenia, epilepsy and diabetic peripheral neuropathy are diseases or disorders which affect millions of people. It is the loss of normal neuronal function which produces the behavioral and physical deficits which are characteristic of each of the different neurological disorders. In addition to chronic and acute neurodegenerative disorders, the aging process and physical trauma to the central nervous system both result in the loss of neural cells accompanied by the associated behavioral and physical deficits. In recent years neurological disorders have become an important concern due to the expanding elderly population which is at greatest risk for these disorders.

Recently, in vitro and in vivo studies have shown that administration of certain agents prior to the occurrence of a neurological insult are capable of exerting a protective effect, resulting in a decrease in neuronal loss. U.S. Patent No. 5,519,035, issued on May 21, 1996, provides a method of treating a patient at risk

for stroke with protein kinase C inhibitor in order to protect neuronal cells from death as a result of cerebral ischemia. Sufficient protein kinase C inhibitor is administered on a daily basis to ensure that, should an ischemic insult occur, the levels of the agent would be high enough to counteract the neurotoxic effects of nitric oxide produced during the ischemic event.

Parkinson's disease, characterized by the loss of dopamine neurons in the nigro-striatal pathway, is a relatively common neurological disorder. The dopaminergic neurotoxin, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) produces parkinsonian symptoms when administered to humans and is frequently used to induce parkinsonionism in animal models of Parkinson's disease. Tomac *et al.* (1995) *(Nature 373* pp 335-339) reported that glial cell line derived neurotrophic factor (GDNF) has a protective effect on dopaminergic neurons when the factor is injected into mouse brain 24 hours before administration of MPTP. Similar results were reported for the neuroprotective effects of GDNF on neurons in the locus coeruleus. Grafting of genetically engineered fibroblasts expressing high levels of GDNF into the locus coeruleus, 24 hours before administration of another dopaminergic neurotoxin, 6-hydroxydopamine (6-OHDA), prevented more than 80% of the 6-OHDA-induced degeneration of noradrenergic neurons in that region (Arenas *et al., Neurons 15*:1465-1473 [1995]). Basic fibroblast growth factor (bFGF or FGF-2) has a protective role for nigrostriatal cells exposed to MPTP or methylpyridiniumion (MPP) (*Park & Mytilineou Brain Res. 599*:83-97 [1992]); Chadi *et al. Exp. Brain Res. 97* :145-158 (1993); Otto and Unsicker *J. Neurosci. Res. 34*:382-393 (1993). Results from in vitro and in vivo studies indicated that FGF-2, administered for a period of up to 4 days before exposure to the neurotoxins and administered for several days after exposure to the toxins significantly decreased the number of dopaminergic neurons damaged by the toxins. Similar results were reported with EGF on cultured cells (Park & Mytilineou, *supra*). In addition, FGF-2 has been shown to have neuroprotective effects in the hippocampus. Koketsu *et al.* (*Ann. Neurol. 35* :451-457 [1994]) reported that FGF-2, administered by continuous intracerbroventricular (icv) infusion for 3 days before and 1 day after focal cerebral ischemia reduced the infarct size produced by the ischemic challenge.

They conclude that the protective effect observed was due to direct trophic action of FGF-2 on neurons, via neuronal gene expression that antagonizes "cell death" programs, or due to trophic effects on glial cells and blood vessels. However, no evidence for glial cell proliferation was observed. Liu *et al. Brain Res. 626*:335-338 (1993) reported that FGF-2, delivered icv for 2 days prior to, and 5 days after intraperitoneal injections of the seizure-inducing neurotoxin kainic acid did not affect the behavioral responses to the toxin but did prevent neuronal cell loss in the hippocampus. They suggest the protective effect of the growth factor on the neurons is due to FGF-2 prevention of the rise in neuronal intracellular calcium levels which normally occurs in response to kainic acid administration.

U.S. Patent 5,438,121, issued on August 1, 1995, relating to brain-derived neurotrophic factor (BDNF), disclosed that BDNF may be added to cell cultures 24 hours prior to exposure to the neurotoxins MPP and 6-OHDA to reduce the amount of cellular damage caused by the neurotoxins. U.S. Patent 5,554,601, issued on September 10, 1996 disclosed a method of protecting cells in the central nervous system from cell death through the stimulation of the production of neurotrophic growth factors in ovarectomized animals. The method relies on the chronic administration of an estrogen compound having a unique structure which allows it to cross the blood brain barrier in order to stimulate the production of neurotrophic growth factor proteins such as nerve growth factor (NGF) and BDNF. The effect of the estrogen compounds was believed to be due to their protective actions against hypoglycemia and excitatory amino acids, and their stimulatory effects on neurotrophic growth factor production in ovarectomized animals. It was noted that the protective effect was not due to a mitogenic effect of the steroid on neural tissue.

International application no. WO 95/13364 discloses the administration of growth factors to neural tissue *in vivo* to induce a patient's stem cells to divide to replace cells that have been damaged as a result of disease.

Summary of the Invention

Methods are provided for protecting mammalian neural tissue from trauma or insult or from the manifestations of neurological disease or disorders or the aging process. The method comprises administering to a mammal an effective amount of one or more growth factors to induce multipotent neural stem cell proliferation which results in a protective effect on the neural tissue. The growth factor treatment can be completed while the neural tissue is still healthy to provided a prolonged protective effect which lasts weeks and even months after cessation of the treatment.

Growth factors such as EGF, amphiregulin, fibroblast growth factor, transforming growth factor alpha, and the like, and combinations thereof can be exogenously administered to the mammal, for example, to one or more CNS ventricles of the mammal. Alternatively, the growth factors can be administered by genetically modifying cells of the mammalian neural tissue to produce the growth factors.

The growth factors are administered at a time prior to the onset of the trauma or the manifestations of the disease or aging process, in amounts sufficient to cause the neural cell population to be protected from progressive cell damage leading to the death of the cells or their malfunction.

The invention is also directed to the use of a growth factor, or a combination of growth factors in the manufacture of a medicament for the preventative treatment of neurological or neurodegenerative diseases or disorders, neurological trauma, and/or the aging of neural tissue.

Description of the Figure:

Fig. 1A-1F depict photomicrographs of Nissl stained coronal sections (10 μm, bregma -3.3 mm) of control (no pretreatment, non-ischemic; 1A, 1B) or one week post-ischemic (1C to 2F) rats that received either vehicle (1C and 1D) or EGF (1E and 1F) i.c.v. infusion t months prior to an ischemic insult. CA1 neurons are present in the non-ischemic controls (1A and 1B) and in the EGF-pretreated ischemic animals (1E and 1F), but are absent in the vehicle-pretreated ischemic

animals (1C and 1D), demonstrating that EGF pretreatment can protect against ischemia-induced cell death in the CA1 region of the hippocampus.

Detailed Description of the Invention:

The present invention provides methods for the protection of neural tissue from the effects of aging, trauma, toxic insult, neurological diseases or disorders. Growth factors are administered to the neural tissue, at a time prior to the onset of trauma or the manifestations of neurological disease or aging, to induce multipotent neural stem cells and neural stem cell progeny to proliferate and generate progeny cells that are capable of differentiating into neurons, astrocytes, and oligodendrocytes. Astrocytes in particular have been shown to provide a supportive and protective role in the brain. These cells buffer the environment around neurons and secrete factors which enhance the survival and function of the neurons. Normally, astrocyte turnover in the CNS is limited and large scale replacement of dysfunctional astrocytes has not been reported.

A "multipotent neural stem cell" is an undifferentiated neural cell which is capable of extensive self renewal, i.e., is capable of replacing itself during cell division over an extended period of time, and is capable of generating the major cell types of the tissues in which it is located (i.e. neurons and glia - astrocytes and oligodendrocytes). The non-stem cell progeny of a neural stem cell are termed progenitor cells. Methods of proliferating neural stem cells *in vitro* and *in vivo* have been previously described (see e.g. WO 93/01275, WO 94/10292, and U.S. Ser. No. 08/486,648).

The term "neural progenitor cell", as used herein, refers to an undifferentiated cell derived from a neural stem cell, and is not itself a stem cell. A distinguishing feature of a progenitor cell is that, unlike a stem cell, it has limited proliferative ability and thus does not exhibit self-maintenance. It is committed to a particular path of differentiation and will, under appropriate conditions, eventually differentiate into either glia (astrocytes or oligodendrocytes) or neurons, and is thus not multipotent.

The term "precursor cells", as used herein, refers to the progeny of neural stem cells, and thus includes both progenitor cells and daughter neural stem cells.

The majority of neural stem cells can be found in the tissue lining the CNS ventricles (including the subependyma) of adult mammals. The term "ventricle" refers to any cavity or passageway within the CNS through which cerebral spinal fluid flows and includes any collapsed portions of the ventricular system. Thus, the term not only encompasses the lateral, third, and fourth ventricles, but also encompasses the central canal, cerebral aqueduct, and other CNS cavities and collapsed CNS cavities.

One or more growth factors may be used to induce the proliferation, migration and/or differentiation of the multipotent neural stem cells/and or their progeny in vivo. As used herein, the term "growth factor" refers to a protein, peptide or other molecule having a growth, proliferative, differentiative, or tropic effect on neural stem cells and/or neural stem cell progeny. Growth factors which may be used for inducing proliferation include any factor that allows neural stem cells and precursor cells to proliferate, including any molecule which binds to a receptor on the surface of the neural stem cell to exert a tropic, or growth-inducing effect on the cell, including any protein, amino acid, vitamin, carbohydrate, or other molecule or atom. Preferred proliferation-inducing growth factors include EGF, amphiregulin, acidic fibroblast growth factor (aFGF or FGF-1), basic fibroblast growth factor (bFGF or FGF-2), transforming growth factor alpha (TGFα), proliferation-inducing ligands which bind to the EGF and FGF receptors, and combinations thereof. A preferred combination of proliferation-inducing growth factors is EGF or TGFα with FGF-1 or FGF-2. To determine whether a particular growth factor will have proliferative effects when administered *in vivo*, the effects of the growth factor on *in vitro* neural stem cell proliferation can be tested using methods already known in the art and disclosed in WO 93/01275, WO 94/10292, and U.S. Ser. No. 08/486,648. Growth factors that induce neural stem cell proliferation *in vitro* have been found to also have proliferative effects *in vivo*.

Experimental results indicate that neural stem cell progeny that have been induced to proliferate *in vivo* by administration of growth factors undergo differentiation into cell types that are beneficial to the treated area. A likely mechanism is that the neural stem cell progeny respond to extrinsic signals that influence them to differentiate into needed cell types. Differentiated progeny of neural stem cells include neurons, astrocytes (type I or type 2) and oligodendrocytes. The differentiative pathway of the neural stem cell progeny can be influenced by the addition of exogenous growth factors that increase the numbers of a particular cell type that are generated. Differentiation-influencing growth factors that can be exogenously added are disclosed in WO 94/10292 and U.S. Ser. No. 08/486,648. Among the growth factors and other molecules that can be used to influence the differentiation of precursor cells are FGF-1, FGF-2, ciliary neurotrophic factor (CNTF), NGF, BDNF, neurotrophin 3, neurotrophin 4, interleukins, leukemia inhibitory factor (LIF), cyclic adenosine monophosphate, forskolin, high levels of potassium, amphiregulin, TGF-α, TGF-ß, insulin-like growth factors, dexamethasone (glucocorticoid hormone), isobutyl 3-methylxanthine, somatostatin, growth hormone, retinoic acid, and PDGF, or ligands which bind to the receptors for these growth factors.

The effects of various growth factors on differentiation can be tested *in vitro* on cultures of multipotent neural stem cell progeny by using dual-label immunocytochemistry various neuronal- and glial-specific antibodies, the effect of the exogenous growth factors on the differentiation of the cells can be determined. Type I astrocytes, which are differentiated glial cells, can be identified by their immunoreactivity for GFAP but not A2B5. Type II astrocytes, which are differentiated glial cells that display a stellate process-bearing morphology, can be identified using immunocytochemistry by their phenotype GFAP(+), A2B5(+) phenotype.

Growth factors can be administered singly or in combination to a patient. They can also be administered in a temporal sequence (e.g. exposure to a first growth factor influences the expression of a second growth factor receptor, Neuron 4:189-201

(1990). The growth factors may be prepared in a pharmaceutically-acceptable excipient. Administration of the growth factors can be done by any method, including injection cannula or injection to the CNS, peripheral injection, timed-release apparati which can administer substances at the desired site, oral administration, and the like. Growth factors can be administered using methods in which the factors may either pass through or by-pass the blood-brain barrier. Methods for allowing factors to pass through the blood-brain barrier include minimizing the size of the factor, or providing hydrophobic factors which may pass through more easily. For example, dimethyl sulfoxide (DMSO) or the like can be administered to reversibly open the blood-brain barrier to allow the passage of intravenously (i.v.) intraperitoneally (i.p.), or orally administered growth factor into the CNS. Doses of DMSO in mice would be from 2-30% DMSO in a volume of approximately 0.25 ml, administered i.v., preferably 10-15%. An intraperitoneal administration of DMSO and growth factor would require from 10-30% DMSO. It would be possible to use Boradeption (Science 217: 166 (1982)) Another possibility would be to bind the growth factor to transferrin to transfer it across the blood brain barrier.

It is presently preferred to administer growth factors directly to one or more ventricles of the CNS. The fact that the majority of neural stem cells are located in the tissues lining ventricles of mature brains offers several advantages for the modification and manipulation of these cells *in vivo*. Treatment can be tailored accordingly so that stem cells surrounding ventricles near the desired region would be manipulated or modified *in vivo* using the methods described herein. The ventricular system is found in nearly all brain regions and thus allows easier access to the desired areas. If one wants to modify the stem cells *in vivo* by exposing them to a composition comprising a growth factor or a viral vector, it is relatively easy to implant a device that administers the composition to the ventricle and thus, to the neural stem cells. For example, a cannula attached to an osmotic pump may be used to deliver the composition. Alternatively, the composition may be injected directly into the ventricles. The neural stem cell progeny can migrate into regions that may be subjected to possible damage as a result of injury, disease, or aging.

Furthermore, the close proximity of the ventricles to many brain regions would allow for the diffusion of a secreted neurological agent by the stem cells or their progeny to the appropriate region.

In addition, or as an alternative to exogenously administering growth factors to precursor cells, the precursor cells can be genetically modified *in vivo* by transfection of the cells with growth factor or hormone-expressing vectors, so that the neural cells express various biological agents useful in the prevention of neurological disorders, trauma and the effects of aging. Methods for genetically modifying multipotent neural stem cells and their progeny are disclosed in published international application no. WO 94/16718. In addition to genetic modification of the cells to express growth factors, the cells may be modified to express other types of neurological agents such as neurotransmitters.

Preferably, the genetic modification is performed either by infection of the cells lining ventricular regions with recombinant retroviruses or transfection using methods known in the art including $CaPO_4$ transfection, DEAE-dextran transfection, polybrene transfection, by protoplast fusion, electroporation, lipofection, and the like [*see* Maniatis et al., Molecular Cloning: A Laboratory Manual (1989), 2nd Ed., Cold Spring Harbor, N.Y.]. Any method of genetic modification, now known or later developed can be used. With direct DNA transfection, cells could be modified by particle bombardment, receptor mediated delivery, and cationic liposomes. When chimeric gene constructs are used, they generally will contain viral, for example retroviral long terminal repeat (LTR), simian virus 40 (SV40), cytomegalovirus (CMV); or mammalian cell-specific promoters such as those for TH, DBH, phenylethanolamine N-methyltransferase, ChAT, GFAP, NSE, the NF proteins (NF-L, NF-M, NF-H, and the like) that direct the expression of the structural genes encoding the desired protein.

Any expression vector known in the art can be used to express the growth factor, as long as it has a promoter which is active in the cell, and appropriate termination and polyadenylation signals. These expression vectors include recombinant vaccinia

virus vectors including pSCll, or vectors derived various viruses such as from Simian Virus 40 (SV40, i.e. pSV2-dhfr, pSV2neo, pko-neo, pSV2gpt, pSVT7 and pBABY), from Rous Sarcoma Virus (RSV, i.e. pRSVneo), from mouse mammary tumor virus (MMTV, i.e. pMSG), from adenovirus(pMT2), from herpes simplex virus (HSV, i.e. pTK2 and pHyg), from bovine papillomavirus (BPV, i.e. pdBPV and pBV-1MTHA), from Epstein-Barr Virus (EBV, i.e. p205 and pHEBo) or any other eukaryotic expression vector known in the art. If a retroviral construct is to be used to genetically modify normally quiescent stem cells, then it is preferable to induce the proliferation of these cells using the methods described herein. For example, an osmotic infusion pump could be used to deliver growth factors to the central canal several days prior to infection with the retrovirus. This assures that there will be actively dividing neural stem cells which are susceptible to infection with the retrovirus.

According to this invention, the deficits in movement, cognition, memory and other behavioral symptoms which normally occur in subjects with neurological disorders caused by disease, trauma or aging or a combination of these factors are reduced as a consequence of preventative administration of a growth factor or growth factors capable of stimulating the proliferation of multipotent neural stem cells and/or their progeny. The growth factor pretreatment method achieves a prolonged protective effect, thereby eliminating the need for the exogenously administered growth factor to be present at the time of the trauma, disease, or disorder. The term "protective effect" means that the growth factor treatment results in a significantly greater number of normally functioning neural cells than would have otherwise been without the growth factor pre-treatment. The growth factors may be administered on an ongoing basis or at regular intervals prior to the manifestations of aging or neurological disorders or disease and from one week to several weeks prior to an anticipated neurological trauma or insult, such as what might occur during brain surgery. As used herein, the term "manifestation" refers to the outward sign of a neurological disease or disorder or the aging process. For example, clinical memory loss is an outward sign of Alzheimer's Disease, and can be an outward sign of the aging process. In contrast, certain biological processes that ultimately lead to

cell death, injury or dysfunction, such as the over- or under-expression of certain gene products, may be early events that begin prior to the onset of outward signs of the aging process, neurological diseases and/or disorders. Diagnosis of these biological processes or other precursor events of the aging process, neurological disease and/or disorder followed by a suitable growth factor treatment may lead to the prevention or reduction of the manifestations of the aging process and neurological diseases and disorders. For example, genetic screening can be used to diagnose patients who are predisposed to certain neurological disorders, such as Huntington's Disease, prior to the onset of manifestations of the disorder. Patients found to be at risk for a neurological disease or disorder can be treated with growth factors to prevent or reduce the progression of the disease.

Growth factors are administered (or neural cells are genetically modified to express growth factors) prior to the onset of trauma or the manifestations of aging or disease processes at concentrations that are sufficient to result in neural stem cell proliferation. The protective effects that are provided by the growth factor treatment are long-term and usually continue for at least one week after treatment has ceased. The protective benefits are often sustained at least two weeks after cessation of treatment, and in many cases, a protective effect is sustained for one, and up to two months, or more.

For the prevention or reduction of the manifestations of Huntington's Disease, Alzheimer's Disease, Parkinson's Disease, and other neurological disorders affecting primarily the forebrain, growth factors or other neurological agents would be delivered to the ventricles of the forebrain to affect *in vivo* proliferation of the stem cells. Alternatively, growth factors and other neurological agents can be easily administered to the lumbar cistern for circulation throughout the CNS. Animal models for various neurological diseases, disorders and injury are used to assess the protective effect of growth factor administration to establish suitable dosages and methods of treatment. Behavioral testing is performed to compare the abilities of growth factor treated animals with non-treated control animals. Such behavioral tests include learning and memory tests such as the Morris water maze and radial

arm maze. The data obtained from accepted mammalian animal models, particularly rodent, canine, and primate models are extrapolated to determine suitable protocols for growth factor treatment of humans. Generally, administration of from about 100 to about 1000 ng growth factor per kilogram of body weight per hour over a period of from about 1 to 10 days is sufficient to induce multipotent neural stem cell proliferation.

Example 2 below demonstrates that epidermal growth factor (EGF), administered icv at a physiologically effective dose, exerts a cytoprotective effect on hippocampal neurons in vivo, preventing the normal neuronal degenerative response following an ischemic insult. While not wishing to be bound by theory, this response appears to be due to the result of the mitogenic effect of the growth factors, resulting in the production of new neural cells, possibly as a result of the induction of proliferation, migration and/or differentiation of quiescent neural stem and progenitor cells and leading to the production of new glial and/or neuronal cells which secrete substances or alter the environment, providing a long term protective effect on the neurons even after the exogenously administered growth factors are no longer present. It is expected that administration of large doses of tritiated thymidine or other anti-mitotic agents at the time of growth factor infusion would block the proliferation of the neural cells, and hence block the protective effect that is observed after ischemic insult. This would indicate that the protective effect observed after growth factor treatment results from the addition of new cells to the area.

The discovery that treatment with growth factors provides a cytoprotective effect can be used advantageously to reduce the chances of or lessen the severity of an expected CNS trauma. For example, a patient scheduled to undergo brain surgery may be pretreated with growth factors to prevent neurological trauma. Prior to surgery, growth factors would be administered to brain regions that may be effected during the surgical procedure. Growth factors are administered at least one week prior to the expected CNS trauma. Preferably, the growth factors would be administered at least two, and more preferably, at least four or more weeks prior to the expected trauma to provide time for the proliferation and differentiation of a

beneficial number of new neural stem cell progeny. If desired, the growth factor pretreatment could be completed several weeks in advance of surgery because the protective effect of growth factor pretreatment is observed for prolonged periods after cessation of the treatment. Certain types of professional athletes, such as boxers, are also at high risk for neurological trauma, and could be treated with growth factors to achieve a neuroprotective effect. Additionally, patients who are at high risk for stroke can be treated with growth factors to reduce the amount of neurological damage should a stroke occur.

Example 3 below demonstrates that in very old animals, the ability to generate an active constitutively proliferating progenitor cell (CPC) population is impaired. Neural precursor populations are shown to decline with age, perhaps as a result of a slowing stem cell cycle time. This in turn may lead to degenerative changes in brain morphology, increased susceptibility to ischemic and toxic insults, and increased frequency of cognitive disorders that are often seen in aged animals. As shown in Example 3, administration of growth factors such as EGF and FGF to the healthy CNS results in the presence of more healthy, young cells in the aging brain. By the phrase "healthy CNS tissue", it is meant that the stem cell cycle time and the neural tissue in general are normal for mammals of the same species and age group. Preferably, at least two weeks after the growth factor treatment, the treated mammal has at least a 5% increase, and more preferably, a 10% increase, in the number of CPCs relative to the average CPC count for age-matched mammals of the same species. The increase in CPC count is sustained for extended time periods, preferably for at least one to two months, and more preferably, for at least four months after cessation of growth factor treatment.

Example 1: In Vivo Proliferation of Neural Stem Cells of Lateral Ventricle

A replication incompetent retrovirus containing the ß-galactosidase gene [as described in Walsh and Cepko, *Science 241*:1342, (1988)] was injected into the forebrain lateral ventricles of CD1 adult male mice (25-30 g from Charles River). The injected retrovirus was harvested from the BAG cell line (ATCC CRL-9560) according to the method of Walsh and Cepko (*supra*). Mice were anesthetized using

65 mg/kg, i.p. sodium pentobarbital. Unilateral stereotactic injections of 0.2-1.0 μl of retrovirus were injected into the lateral ventricle using a 1 μl Hamilton syringe. The coordinates for injection were AP +4.2 mm anterior to lambda, L ± 0.7 mm, and DV -2.3 mm below dura, with the mouth bar at -2mm below the interaural line.

One day, or six days following the retrovirus injection, an infusion cannulae attached to a 0.5 μl/hour ALZET osmotic mini-pumps filled with 3.3 - 330 μg/ml of EGF were surgically implanted into the lateral ventricles at the identical stereotactic coordinates as stated above. The infusion cannula kits were obtained from ALZA. The infusion cannulae were cut to 2.7 mm below the pedestal. The pumps were secured to the mouse skull by use of acrylic cement and a skull screw contralateral and caudal to the injection site. The osmotic mini-pump was situated subcutaneously under and behind the armpit of the left front paw and connected to the infusion cannula by the means of polyethylene tubing.

Six days following initiation of EGF infusion the animals were sacrificed with an overdose of sodium pentobarbital. Mice were transcardially perfused with 2% buffered paraformaldehyde, and the brains were excised and post fixed overnight with 20% sucrose in 2% buffered paraformaldehyde. Coronal slices were prepared with -20 Celsius cryostat sectioning at 30 μm. Slices were developed for ß-gal histochemistry as per Morshead and Van der Kooy (*supra*).

Under these conditions, regardless of the day post retrovirus injection, infusion of EGF resulted in an expansion of the population of ß-gal labeled cells from an average of 20 cells per brain up to an average of 150 cells per brain and the migration of these cells away from the lining of the lateral ventricles. Infusion of FGF-2 at 33 μg/ml resulted in an increase in the number of ß-gal labeled cells, but this increase was not accompanied by any additional migration. Infusion of EGF and FGF together resulted in an even greater expansion of the population of ß-gal labeled cells from 20 cells per brain to an average of 350 cells per brain. The synergistic increase in ß-galactosidase cell number when EGF and FGF are infused together further reflects the direct association between the relatively quiescent stem

cell and the constitutively proliferating progenitor cell.

This experiment can be modified to infuse growth factors in other CNS ventricles to achieve a similar expansion of cell numbers in other CNS regions.

Example 2: Growth Factor Infusion Prior to Neurological Insult

Epidermal Growth Factor (EGF) (Chiron Corp.) was prepared in 1 mg/ml rat albumin (Calbiochem, Cat. No. 126722) in sterile physiological saline. The EGF preparation was infused into the rostral lateral ventricle (Bregma +0.7 mm) of adult male Wistar rats weighing 250- 350 g via a 30 gauge cannula connected to an osmotic minipump (Alza, Model 2001) at a rate of 1 μl/hr for 9 days. The EGF was prepared at a concentration so as to provide each animal with 416 ng EGF per kilogram of body weight every hour.

Precursor Cell Labeling

Animals received BrdU (50 mg/kg) i.p. 3 times per day for the last 3 days of growth factor infusion.

Ischemia Lesions

The model used was a combination of rodent models used routinely for transient global ischemia which combines bilateral common carotid artery occlusion with hemorrhagic hypotension (Smith *et al.* Act. Neurol. Scand 69:385-401 (1984); Mudrick and Baimbridge, Exp'l Brain Res. 86:233-247 (1991)). This model produced lesions of the neurons in the CA1 region (100% of animals) and in the CA2 and CA3 regions (60% of animals) of the hippocampus bilaterally. Two months after treatment with EGF, the animals were anesthetized with sodium pentobarbital (60 mg/kg i.p.) and treated with atropine (0.2 mg/kg i.p.) to reduce respiratory secretions. A rectal temperature probe, connected to a temperature regulated heat pad, was used to maintain the animal body temperature at 35°C. The common carotid arteries and the femoral artery were isolated using blunt dissection and a 3-0 silk was looped around each vessel for later access. The femoral artery was then cannulated and heparin (150 units) was administered to prevent clotting.

The baseline mean arterial pressure was taken (~120/80 mmHg) via a transducer and recorded on a chart recorder. The animal was hemorrhaged until mean arterial pressure fell to 30-40 mmHg (removal of approximately 7-10 ml of blood). The common carotid arteries were quickly occluded with atraumatic arterial clamps for 20 minutes. Mean arterial pressure was maintained at 30-40 mmHg throughout the duration of the ischemia by either further hemorrhage or reinfusion of blood. Blood flow was restored by removal of the arterial clamps, and reinfusion of shed blood. Protamine sulfate (1.5 mg/animal) was administered via the femoral cannula to deactivate heparin to prevent post-surgical bleeding. Mean arterial pressure should return to pre-ischemic levels or slightly higher. The animal was given 5 ml of lactated Ringers s.c. and allowed to recover under a warm lamp. The animal was single-housed and monitored closely for the next 48 hours.

Tissue Preparation

Animals were allowed to survive 1 week after the ischemic insult prior to tissue preparation. The animals were deeply anesthetized with 65 mg/kg sodium pentobarbital and transcardially perfused with ice cold saline (~350 ml) followed by ice cold 4% paraformaldehyde (PFA) in 0.1M phosphate buffer at pH 7.4 (~500ml). Brains were removed and post fixed in 4% PFA overnight at 4°C. The brains were then cryoprotected through serial overnight incubations in 10% to 30% sucrose at 4°C and then incubated for 2-24 hours in 2:1 30% sucrose:OCT. The brains were then frozen on dry ice and 10μm serial sections were cut on a cryostat.

Histological Evaluation of Neuronal Cell Death

Tissue processed for morphology was defatted in Histoclear (Diamed, Cat. No. HS-200), rehydrated through a descending series of alcohols, rinsed in distilled water and stained with Thionin (0.5%). Tissue was then dehydrated through a series of alcohols, cleared with Histoclear, and coverslipped with DPX (Aldrich, Cat. No. 31761-1). Thionin stains the Nissl substance within the cytoplasm of nerve cells (Cajal, 1995). The absence of cells with the characteristic pyramidal cell morphology within the cell layer is indicative of a loss of hippocampal pyramidal neurons due to ischemia-induced cell death. A one week post ischemia survival time

was chosen due to the unique characteristic of delayed neuronal death observed in CA1 pyramidal cells in response to transient global ischemia (Shigeno *et al.* J. Neurosci. 11:2914-2919 (1991); Ordy *et al.* Exp'l Neurol. 119:128-139 (1993)) whereby select CA1 pyramidal cells appear unaffected by ischemia for up to 3 days post surgery. In addition, maximal glial reaction has occurred and excitotoxic conditions have subsided by this time point.

Results

EGF pretreatment (icv infusion of EGF for a 9 day period) given 8 weeks prior to an ischemic insult produced complete protection in 3 of 7 animals from ischemia-induced neuronal cell death in the CA1 region. Nissl stain of EGF-treated animals revealed that the cells of the CA1, CA2 and CA3 displayed excellent morphology throughout the extent of the hippocampus (Figure 1). In addition, there was an increased number of glial cells observed within the hippocampus. BrdU labeling was observed within the hippocampus.

Example 3: Growth Factor Administration Prior to Onset of Advanced Aging

Assessment of precursor cells in rodents of various ages

The adult subventricular zone (SVZ) consists of at least two populations of dividing cells that can be distinguished by differences in their cell cycle times: a slowly cycling stem cell population and a rapidly cycling, constitutively proliferating progenitor cell (CPC) population. The purpose of this study was to quantify the numbers and distribution of these cell types with age in the mouse and rat and the impact of growth factor infusion on cell numbers.

Mice of various ages (between 2 and 18 months) were injected with BrdU (approximately 67 mg/kg) for 4 weeks (3-5 times/day) and left undisturbed for a further 8 weeks. This paradigm resulted in labeling of the slowly cycling stem cells of the subventricular zone. Animals were perfused with 4% paraformaldehyde and the brains are removed and cryoprotected. Frozen sections were cut at 30 μm. BrdU was detected immunocytochemically (rat anti-BrdU, Sera labs, followed by donkey anti-rat CY3 or FITC, Jackson Immunoresearch). Labeled cells throughout

the brain were quantified and changes in cell numbers or distribution with age were analyzed. In addition, the distribution of BrdU-immunoreactive, post-mitotic stem cell progeny in the brain was quantified and compared among ages.

The same tissue was double-labeled with antibodies to proliferating cell nuclear antigen (PCNA), which is upregulated around the S phase of the cell cycle. Preliminary results indicate that it marks the constitutively proliferating cell population. The number and distribution of PCNA-IR cells were quantified and compared among ages.

Results

The number of mitotically active stem cells (BrdU-IR) declined with age, particularly at stem cell "pockets" where there are higher concentrations of stem cells. At the rostral stem cell pocket (bregma 0.7), counts for the 7 month old group and the 13 month old animal were 65% and 53% of the 2 month values, respectively. Similarly, at the caudal stem cell pocket (bregma -4.3), counts for the 7 month and 13 month groups were 53% and 38% of the 2 month values, respectively.

The CPC population (PCNA-IR) also declined with age. The ages of the animals at the time of sacrifice for CPC analysis were 5, 10, and 16 months. To avoid confusion when comparing data, the age at which the stem cell labeling began (i.e. 2, 7 and 13 months) is used to identify the subset of animals referred to (rather than the age at sacrifice). At bregma 0.7, a previously identified CPC peak, counts for the 7 month group were only 62% of the 2 month values, while the 13 month animal contained only 30% of the 2 month counts. This data contradicts that of Kuhn *et al.* J. Neuro Sci 16:2027-2033 (1996), who found no change in the number of BrdU-IR cells with age in a restricted area of the rat lateral ventricle when analyzed 1 day after BrdU injection.

The number of BrdU-IR cells in the dentate gyrus of the hippocampus also declined with age.

Effect of growth factors on the aging process of rodents

EGF was infused into the lateral ventricles of two month old adult CD1 mice (n=3), using the procedure described in Example 2 for a period of 6 days. Control mice (n=3) were infused with vehicle. The animals were sacrificed four months later. The number of PCNA-IR cells around the lateral ventricle was measured, using the procedures described above, in 10 μm sections from the following bregmas: 2.7, 2.2, 1.7, 1.2, 0.7, 0.2, -0.3, -0.8. These bregma correspond to the peak cell distributions of CPCs and are the sites where significant loss of PCNA-IR cells with age are seen. Two of the three animals that received EGF treatment had significantly higher PCNA counts (approx. 30% higher) than vehicle controls demonstrating that the EGF has a protective effect on the CPCs in aging animals.

In further studies, EGF-treated animals are allowed to age and are given a variety of memory and other behavioral tests and compared with animals of the same age that have not received EGF treatments. Improved performance of EGF-treated animals compared to non-treated animals demonstrates that pretreatment with growth factors can protect against or reduce age-related declines in cognitive function.

Example 4: Growth Factor Administration Prior to Onset of Neurological Disease

Parkinson's Disease Model

Animals receive EGF infusion treatments as described in Example 2 for 6 days (mice) or 9 days (rats). After various time points (1 wk, 2 wk, 1 mo., 2 mos., 4 mos. and 8 mos.) after EGF treatment, animals are lesioned so as to model Parkinson's Disease. In rat models, lesions are made by knife-cuts of the dopaminergic fibers projecting from the midbrain to the striatum or by one injection of 8 μg of 6-hydroxydopamine directly into the striatum, nucleus accumbens or the substantia nigra. In mice, lesions are made by administration of two injections at 16 hours interval, of 50 mg MPTP/Kg subcutaneously in volume of 0.5 ml of 0.9% saline solution. A subset of animals are allowed to survive one to eight days after lesioning and are then sacrificed. The numbers of surviving dopaminergic cells are assayed by tyrosine hydroxylase (TH) immunocytochemistry and are compared with

control animals that are lesioned prior to sacrifice but which do not receive growth factor treatment. Another subset of animals that are allowed to survive for prolonged periods and are subjected to behavioral testing and compared with lesioned controls animals (no growth factor treatment) or normal animals that are the same age as the model animals.

Alzheimer's Disease Model

Female Wistar rats (180-200 g) are used for this model. The animals receive EGF infusion treatments as described in Example 2 for 9 days. After various time points (1 wk, 2 wk, 1 mo., 2 mos., 4 mos. and 8 mos.) after EGF treatment, animals are lesioned so as to model Alzheimer's Disease. A retractable wire knife (Scouten Knife) is used to partially lesion the fimbria-fornix pathway. Animals are killed at various time points after the lesion (from 1 day to 8 weeks), and the numbers of surviving cells are assayed by Nissel staining in the septum. Controls, lesioned and treated animals are compared. A subset of animals is allowed to survive for prolonged periods for behavioral studies and compared with control animals.

Huntington's Disease Model

Following pretreatment with administered growth factors as described in Example I, the method described Levivier et al (1995) *Neurosci.* 69(1):43-50 is used to induce quinolinic acid lesions in striatal tissue. The numbers of neurons in the striatum are compared between controls, lesioned and treated animals.

WHAT IS CLAIMED IS:

1. A method of preventing the decline of the constitutively proliferating neural progenitor cell population of mammalian neural tissue in an aging mammal comprising administering to a mammal an effective amount of one or more growth factors to induce multipotent neural stem cell proliferation and provide a protective effect on said constitutively proliferating neural progenitor cell population.

2. The method of claim 1 wherein said neural tissue is healthy.

3. The method of claim 1 wherein said one or more growth factors is selected from the group consisting of EGF, amphiregulin, acidic fibroblast growth factor (aFGF or FGF-1), basic fibroblast growth factor (bFGF or FGF-2), transforming growth factor alpha (TGFα), proliferation-inducing ligands which bind to the EGF and FGF receptors, and combinations thereof.

4. The method of claim 1 wherein said one or more growth factors is administered to one or more ventricles of said mammal.

5. The method of claim 1 wherein said one or more growth factors is administered by genetically modifying cells of said mammalian neural tissue to produce said one or more growth factors.

6. A method of protecting a mammalian neural tissue against neurological trauma or insult comprising administering to said mammal an effective amount of one or more growth factors to induce multipotent neural stem cell proliferation and provide a protective effect on said neural tissue at a time prior to an anticipated neurological insult or trauma.

7. The method of claim 6 wherein administration of said growth factor to said mammal is completed at least one week prior to said neurological insult or trauma.

8. The method of claim 6 wherein said anticipated neurological insult or trauma is due to surgical procedures performed on said mammal's brain.

9. The method of claim 6 wherein said one or more growth factors is selected from the group consisting of EGF, amphiregulin, acidic fibroblast growth factor (aFGF or FGF-1), basic fibroblast growth factor (bFGF or FGF-2), transforming growth factor alpha (TGFα), proliferation-inducing ligands which bind to the EGF and FGF receptors, and combinations thereof.

10. The method of claim 6 wherein said one or more growth factors is administered to one or more ventricles of said mammal.

11. A method of preventing or reducing the manifestations of a neurological disease or disorder in a mammal comprising administering to said mammal an effective amount of one or more growth factors to induce multipotent neural stem cell proliferation at a time prior to occurrence of said manifestations.

12. The method of claim 11 wherein prior to administration of said one or more growth factors to said mammal, said mammal is diagnosed with a precursor event to said manifestations of said neurological disease or disorder.

13. The method of claim 11 wherein said one or more growth factors is selected from the group consisting of EGF, amphiregulin, acidic fibroblast growth factor (aFGF or FGF-1), basic fibroblast growth factor (bFGF or FGF-2), transforming growth factor alpha (TGFα), proliferation-inducing ligands which bind to the EGF and FGF receptors, and combinations thereof.

14. The method of claim 11 wherein said one or more growth factors is administered to one or more ventricles of said mammal.

15. The method of claim 11 wherein said one or more growth factors is administered by genetically modifying cells of said mammalian neural tissue to

produce said one or more growth factors.

CONTROL

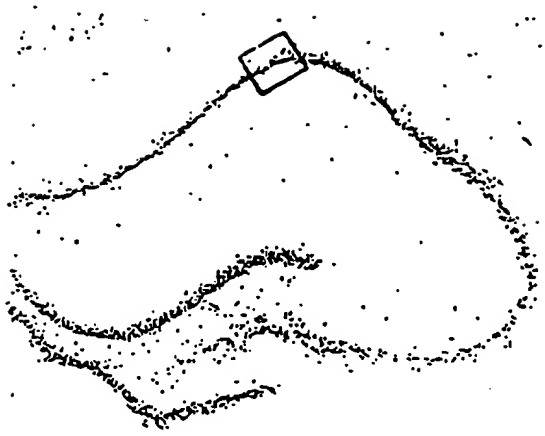

FIG._1A

CONTROL

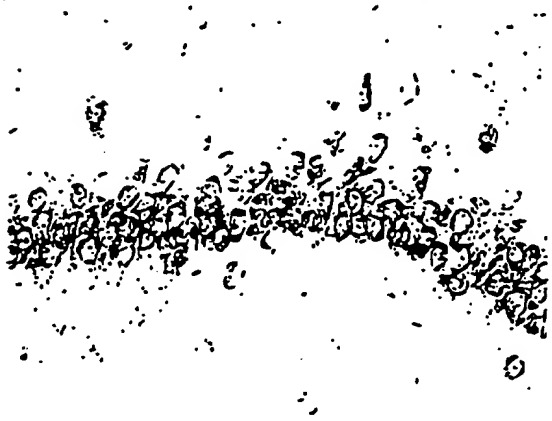

FIG._1B

VEHICLE

FIG._1C

VEHICLE

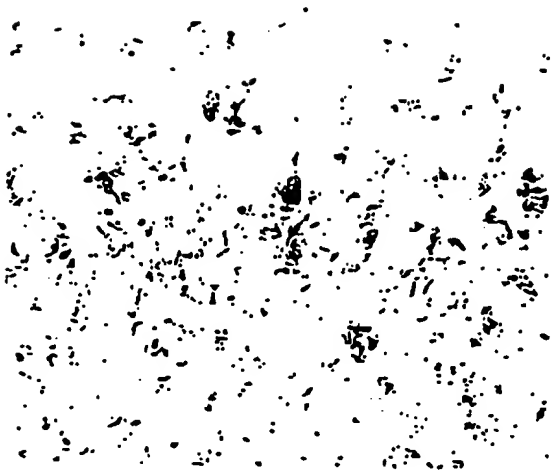

FIG._1D

EGF

FIG._1E

EGF

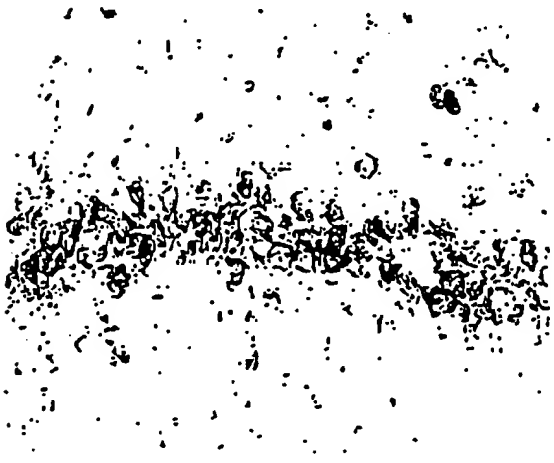

FIG._1F

INTERNATIONAL SEARCH REPORT

Intern ial Application No
PCT/CA 97/00859

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K38/18 A61K48/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 95 13364 A (NEUROSPHERES LTD ;WEISS SAMUEL (CA); REYNOLDS BRENT A (CA); KOOY D) 18 May 1995 cited in the application see page 5, line 24 - page 7, line 2	1-15
A	WO 96 15226 A (NEUROSPHERES HOLDINGS LTD ;WEISS SAMUEL (CA); REYNOLDS BRENT A (CA) 23 May 1996 see page 8, line 26 - page 9, line 19	1-15
P,A	WO 97 35605 A (NEUROSPHERES HOLDINGS LTD) 2 October 1997 see page 3, line 15 - page 4, line 28	1-15

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

1

Date of the actual completion of the international search	Date of mailing of the international search report
17 March 1998	08.04.98
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Authorized officer Sitch, W

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA 97/ 00859

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 1-15
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claim(s) 1-15 is(are) directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Interr. ...nal Application No
PCT/CA 97/00859

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9513364 A	18-05-95	AU 8056194 A	29-05-95
		CA 2175992 A	18-05-95
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		EP 0728194 A	28-08-96
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		JP 9507747 T	12-08-97
		NO 961859 A	03-07-96
WO 9615226 A	23-05-96	AU 3836795 A	06-06-96
		EP 0792350 A	03-09-97
		FI 971956 A	04-07-97
		NO 972171 A	07-07-97
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